A cell-based system that recapitulates the dynamic light-dependent regulation of the vertebrate clock

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Edited by Solomon H. Snyder, Johns Hopkins University School of Medicine, Baltimore, MD, and approved July 6, 2001 (received for review May 9, 2001)

The primary hallmark of circadian clocks is their ability to entrain to environmental stimuli. The dominant, and therefore most physiologically important, entraining stimulus comes from environmental light cycles. Here we describe the establishment and characterization of a new cell line, designated Z3, which derives from zebrafish embryos and contains an independent, light-entrainable circadian oscillator. Using this system, we show distinct and differential light-dependent gene activation for several central clock components. In particular, activation of *Per2* expression is shown to be strictly regulated and dependent on light. Furthermore, we demonstrate that *Per1*, *Per2*, and *Per3* all have distinct responses to light–dark (LD) cycles and light-pulse treatments. We also show that *Clock*, *Bmal1*, and *Bmal2* all oscillate under LD and dark–dark conditions with similar kinetics, but only *Clock* is significantly induced while initiating a light-induced circadian oscillation in Z3 cells that have never been exposed to a LD cycle. Finally, our results suggest that *Per2* is responsible for establishing the phase of a circadian rhythm entraining to an alternate LD cycle. These findings not only underscore the complexity by which central clock genes are regulated, but also establishes the Z3 cells as an invaluable system for investigating the links between light-dependent gene activation and the signaling pathways responsible for vertebrate circadian rhythms.

In recent years, a new and exciting dimension has been added to our knowledge of the vertebrate circadian clock system. The classical view of the circadian system describes it as diverse physiological rhythms, which are regulated by a centralized clock structure (1, 2). Over the past few years, the idea that the clock consists exclusively of a few centralized structures has been challenged. Data coming from both vertebrate and invertebrate systems have demonstrated that the circadian timing system is dispersed throughout the animal (3–7), and that possibly every cell contains a functional circadian clock (8). In these studies, it has been revealed that a variety of tissues and cells contain functional autonomous clocks. These clocks are able to maintain an oscillation when placed *in vitro* and removed from any external cues or signals that originate from the classical clock structures and/or the environment.

The discovery of a number of genes involved in the generation and maintenance of circadian oscillations (9, 10) and the recent realization that the circadian system consists of a complex network of independent clocks, which are somehow synchronized to properly regulate all physiological rhythms (5, 11, 12), has necessitated the development of new tools and methodologies for deciphering the circadian system. An ideal tool is an *in vitro* cell-based system that displays robust circadian rhythms. Cultured cells may be used to fully understand the complex molecular mechanisms, signal coupling, and regulatory feedback loops that are responsible for the proper timing of a circadian oscillation. A remarkable example of such a system is the serum shock-induced circadian oscillation that can be initiated in immortalized cells (8). The physiological relevance of this system was bolstered by the demonstration that an *in vitro* rhythm in cultured rat liver and lung could be reinitiated by a simple medium change (5). The serum shock system is ideal for looking at oscillating RNA and protein levels, protein localization, and posttranslational modifications that play a role in maintaining an oscillation (8). However, this system is unable to offer any assistance in the study of light-dependent signaling and mechanisms that regulate circadian rhythms.

We have previously demonstrated that the zebrafish is a very attractive system for studying light-dependent circadian rhythmicity *in vitro* (3, 4, 13). The ability to look at light-dependent signaling, oscillation, and entrainment has yet to be accomplished by any *in vitro* mammalian system. These are all aspects of the vertebrate circadian system that can be studied in zebrafish-based *in vitro* systems. In the present study, we describe a newly developed, highly light-responsive, zebrafish cell line that displays robust and tightly regulated circadian oscillations. In this cell line, designated Z3, we observe distinct and differential light-dependent gene activation for several central clock components. The gene expression profiles displayed under various light cycles and conditions further elucidate specific light-dependent regulation of clock genes and their functions.

Materials and Methods

Fish and Embryo Harvest. Zebrafish were fed twice daily and kept at 29°C. They were maintained under a 14-h day, 10-h night cycle. Mating tanks were set up just before the beginning of the night phase. Embryos were collected the following morning, just after the beginning of the day phase. The collected embryos were kept at 29°C and allowed to age for ≈24 h.

Cell Culture. Embryo isolation, dispersion, and culture preparation was done by using the following procedure. The 24-h embryos were rinsed in 0.5% bleach for 2 min and then rinsed three times in sterile PBS. After the final rinse in PBS, embryos were manually dechorionated by using sterile forceps. Dechorionated embryos were then transferred to a tissue culture hood and placed in a sterile beaker containing sterile PBS and rinsed two times for 5 min. The PBS from the final wash was removed, and the embryos were dissociated by placing the embryos in 0.25% trypsin at a concentration of 50 embryos/ml and incubating them at room temperature. Trypsinization was accompanied by manual dispersion by pipetting the embryos 5–10 times through a P1000 (Gilson) every 3–5 min. Dissociation was continued until mostly single cells were obtained. The cell suspension was rinsed two times in 10 ml of L15 medium supplemented with 15% FCS, 2 mM glutamine, gentamycin, streptamycin, and penicillin (GIBCO/BRL). After the final rinse in L15, the cells were spun down at 300 × *g* and resuspended in complete L15 medium at a concentration of 20 embryos/ml. Then 5 ml of the resuspended culture was placed

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: Bmal, brain and muscle ARNT-like; CT, circadian time; Cry, cryptochrome; DD, dark–dark; LD, light–dark; Per, period; ZT, Zeitgeber time.

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 www.pnas.org/cgi/doi/10.1073/pnas.181228598

in sealed 25-cm^2 flasks and placed in an incubator maintained at 25°C. Cultures were replated at a 1:2 dilution once reaching confluence. Over the first several passages, a subpopulation able to survive under the given culture conditions grew out and established the Z3 line.

RNA Analysis. Total RNA extraction was done by using RNA-Solv (Omega Bio-tek, Doraville, GA) as recommended by the manufacturer. A miniaturized RNase protection assay was performed as described (3). RNA was equilibrated on agarose gels by ethidium bromide staining. The zebrafish *Clock*, *Bmal1*, and *Bmal2* probes for RNase protection assay were generated as described (3, 13). Zebrafish *Per1*, *Per2*, and *Per3* probes corresponding to nucleotides 1–223, 1981–2369, and 1218–1572 of each respective ORF were used, and all riboprobes were made by using an *in vitro* transcription kit (Promega). Each point was prepared from one confluent 25-cm^2 flask of Z3 cells.

Lighting and Temperature Control. Cultured flasks were kept in a water-jacketed, thermostatically controlled, and light-sealed incubator. Illumination was achieved by using a halogen light source fed into the incubator through a fiber optic line. A programmable timer connected to the light source controlled the light cycles. All light–dark (LD) cycles consisted of 12 h of light and 12 h of dark, unless otherwise noted.

Results

Oscillating Gene Expression in Z3 Cells Exposed to a LD Cycle. Recent findings (4) demonstrate that peripheral tissues in the zebrafish are directly light-responsive *in vitro*. With the aim of creating a cell-based system to study the molecular mechanisms that regulate light-entrainable circadian rhythms, we established a new zebrafish cell line designated Z3. The Z3 cell line was derived from 24-h-old zebrafish embryos and maintained in constant darkness. To determine whether or not the Z3 cells were light-responsive, we placed them on a 12:12 LD cycle for 5 days before harvesting RNA to study possible oscillations in gene expression. Strikingly, our analysis shows that many of the known clock components display a robust rhythmic oscillation (Fig. 1).

The *Per* genes all have a robust oscillation under LD conditions (Fig. 1). *Per1* reaches peak expression level at Zeitgeber time (ZT) 0, where ZT0 corresponds to lights-on and ZT12 to lights-off. *Per2* has maximal expression at ZT3, whereas *Per3* peaks at ZT3–6. It is evident that all three *Per* genes have varied regulatory mechanisms. This data is not only demonstrated by the differences in the timing of their maximal expression, but also by the dynamics of their expression patterns. *Per1* and *Per3* are able to anticipate dawn as the expression of both genes is activated before lights-on (Fig. 1, lane 9). On the other hand, *Per2* does not exhibit expression before the initiation of the light phase. Finally, although *Per1* and *Per3* are down-regulated with similar kinetics, *Per2* lags just behind, leaving the expression of all three genes significantly down-regulated by the beginning of the dark phase.

As we have previously observed in zebrafish peripheral tissues (3, 4, 13), *Clock* and its partners *Bmal1* and *Bmal2* also have oscillating gene expression under LD conditions in the Z3 cell line (Fig. 1). *Clock*, *Bmal1*, and *Bmal2* all have similar oscillation kinetics under LD conditions. All three genes reach peak expression at ZT18 and are down-regulated to basal levels before lights-on. Interestingly, there seems to be a modest rebound in *Clock*, *Bmal1*, and *Bmal2* expression at ZT0 that is suppressed by ZT3. Thus, under LD conditions, the Z3 cells are able to detect and translate a LD cycle into the robust circadian oscillation of central clock components. This result demonstrates that the Z3 cells not only contain the appropriate phototransducing molecules to receive and transmit a photic signal, but that they also contain a functional circadian oscillator.

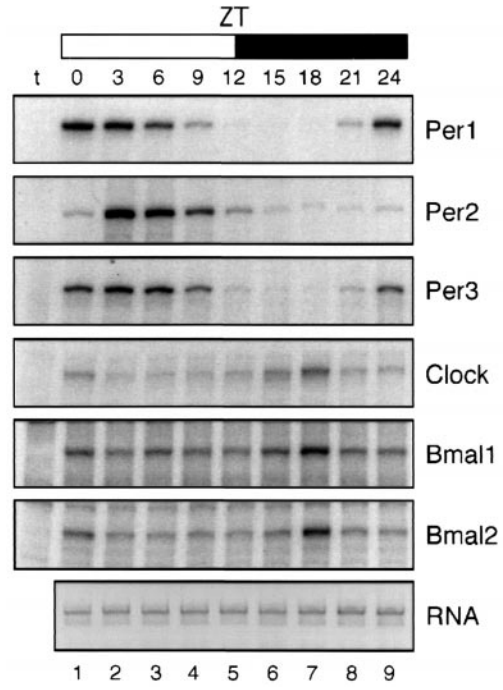

Fig. 1. Oscillation of clock components under a LD cycle. RNase protection analysis of *Per1*, *Per2*, *Per3*, *Clock*, *Bmal1*, and *Bmal2* gene expression in Z3 cells entrained for 5 days under a 12:12 LD cycle. RNA was harvested at the indicated ZTs during day 6 of the LD cycle. The bar above indicates light (white) and dark (black) periods. tRNA serves as a negative control reaction (t). Relative amounts of total RNA used for each sample are displayed (RNA). The ZT0 and ZT24 represent two independent samples that were harvested at the represented dark to light transition points.

Oscillating Gene Expression in Z3 Cells Is Conserved in Constant Darkness. We next wanted to know whether the rhythmic gene expression observed under LD conditions persisted through a dark–dark (DD) cycle. Fig. 2 shows that gene expression continues to oscillate under DD for all genes examined except *Per2*. This striking observation demonstrates that *Per2* expression is strictly light-dependent and that its transcriptional regulation is uncoupled from that of *Per1* and *Per3*. Interestingly, differential regulation of *Per1* and *Per2* expression and the lack of *Per2* oscillation under DD conditions also have been observed in the *Xenopus laevis* retina (14). Under DD conditions, *Per1* and *Per3* continue to oscillate, although not as tightly as in the LD cycle. Expression of *Per1* and *Per3* spans a large proportion of the cycle and displays an increase in basal expression. The maximal expression of *Per1* shifts forward from ZT0, in LD (Fig. 1), to circadian time (CT) 3 in DD (Fig. 2). The peak expression of *Per3* also shifts to CT3 in DD (Fig. 2), slightly earlier than its peak of ZT3–6 under LD conditions (Fig. 1). Both, *Per1* and *Per3* are still able to anticipate dawn, as is evidenced by their increased expression before lights-on (Fig. 2, lane 9).

Clock oscillation is sustained under DD conditions, but its peak is spread more between CT15 and CT18 (Fig. 2). Peak expression of *Bmal1* and *Bmal2* also falls closer to CT15 (Fig. 2) than was observed under a LD cycle (Fig. 1). The slight readjustment of each of the remaining oscillating genes in their timing of maximal gene expression may be a consequence of the total loss of *Per2* oscillation in DD. Therefore, we conclude that expression of *Per2* is strictly light-dependent and that the Z3 cells are able to sustain a rhythmic cycle in the absence of any photic stimulation.

Entrainment Kinetics of Naive Z3 Cells to a LD Cycle. To further characterize the molecular mechanisms and regulatory loops

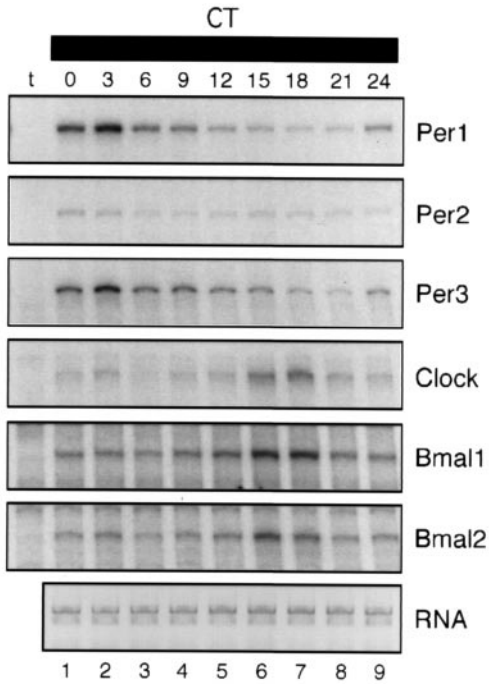

Fig. 2. Oscillation of clock components under a DD cycle. RNase protection analysis of *Per1*, *Per2*, *Per3*, *Clock*, *Bmal1*, and *Bmal2* gene expression in Z3 cells entrained for 5 days under a 12:12 LD cycle and then placed in constant darkness. RNA was harvested at the indicated CTs during the first full day of a DD cycle. The bar above indicates the DD (black) period. tRNA serves as a negative control reaction (t). Relative amounts of total RNA used for each sample are displayed (RNA).

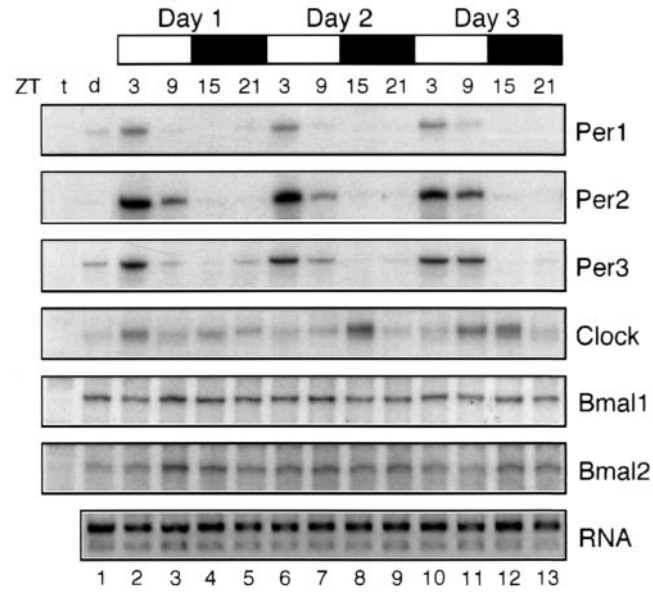

Fig. 3. Entrainment of naive Z3 cells to a LD cycle. RNase protection analysis of *Per1*, *Per2*, *Per3*, *Clock*, *Bmal1*, and *Bmal2* gene expression in Z3 cells maintained in constant darkness and never before exposed to a LD cycle. Naive cells were exposed to a 12:12 LD cycle, and RNA was harvested at ZT3, 9, 15, and 21. A flask of cells, synchronously prepared with the experimental samples, was continuously maintained in total darkness and harvested at the same time as the ZT3 sample of day 1, representing our dark control (d). The bar above indicates light (white) and dark (black) periods. tRNA serves as our negative control reaction (t). Relative amounts of total RNA used for each sample are displayed (RNA).

that are present in the Z3 cells, we evaluated the entrainment kinetics of naive (cells that have never been exposed to a LD cycle) Z3 cells to a LD cycle. The *Per* genes are able to entrain to the new cycle immediately and maintain a precise high amplitude oscillation throughout the course of the experiment (Fig. 3). When comparing ZT3 of day 1 and the DD control sample, it is evident that all three *Per* genes are strongly activated by the initiation of a LD cycle (compare lanes 1 and 2). This demonstrates that during the initiation of a light-dependent rhythm in naive Z3 cells that all three *Per* genes are light-responsive and likely play a role in setting the phase of the newly initiated rhythm.

As with the *Per* genes, a significant induction of *Clock* expression is seen after the initiation of the light phase (Fig. 3, compare lanes 1 and 2). This initial increase in expression is quickly suppressed, possibly because of the simultaneous expression of the *Per* genes (compare *Clock* lanes 2 and 3). A moderate rebound in expression is seen at ZT15 of day 1 (Fig. 3, lane 4), which is the normal time of *Clock* peak expression, and then a robust ZT15 peak is established for days 2 and 3. As for *Bmal1* and *Bmal2*, both follow very similar expression profiles. Significant activation of the *Bmal* genes does not occur immediately after the initial light phase but is delayed ≈6 h with respect to what is observed for the *Per* genes and *Clock* (Fig. 3, lanes 1–3). Both *Bmal* genes gradually entrain to a ≈ZT15 peak by day 3 of the LD cycle. Because *Bmal1* and *Bmal2* reach maximal expression at ZT18 under LD cycling (Fig. 1), it is more difficult to see a clear rhythm for the *Bmal* genes with the time points analyzed (Fig. 3). However, although the *Per* and *Clock* genes are already rhythmic by days 1 and 2, respectively, it appears that *Bmal1* and *Bmal2* do not entrain to the new rhythm until day 3 of the cycle. This result suggests that *Bmal* oscillation is not essential for entrainment of a newly initiated rhythmic oscillation. Therefore, *Clock* and the *Per* genes are more actively responsive, and play a more critical role, in the entrainment of a newly initiated light-dependent circadian oscillation in naive Z3 cells.

Sustainability of an Established Circadian Rhythm in Constant Darkness. To ascertain whether circadian rhythmicity could persist under constant darkness, we subjected Z3 cells that were entrained for 5 days on a 12:12 LD cycle, to several days of DD (Fig. 4). As previously shown (Fig. 2), *Per2* expression immediately ceases to oscillate in the absence of light stimulation (Fig. 4). *Per1* and *Per3* are able to maintain an accurate rhythm through 2 days of constant darkness. The accuracy of the rhythm begins to be lost on day 3 of DD, as seen by a shift in peak expression from CT3 to CT9 (Fig. 4, lane 10). By day 4 of DD, all resemblance to the original rhythm is lost.

Clock, *Bmal1*, and *Bmal2* are also able to maintain an accurate rhythm through day 2 of DD (Fig. 4). Rhythmic accuracy is lost during day 3 of constant darkness, and a forward shift of 6 h is observed in peak expression, from CT15 to CT21 (Fig. 4, lane 12). In similar but separate independent experiments, the pattern of peak expression for all genes examined is consistent throughout the initial loss of rhythm accuracy that occurs during day 3 of constant darkness and is characterized by a 6-h shift forward in peak expression. However, the expression peaks observed during subsequent days of DD occur at random intervals when compared between experiments. Therefore, the Z3 cell line is able to maintain a precise circadian rhythm for 2 cycles of DD, accuracy is lost during day 3, and expression patterns are randomized by day 4 of constant darkness.

Re-Entrainment of Z3 Cells to a Cycle Inversion. To determine which clock component(s) might be responsible for setting the new phase of an oscillation entraining to an alternate LD cycle, we subjected the Z3 cells to a cycle inversion that created a 12-h

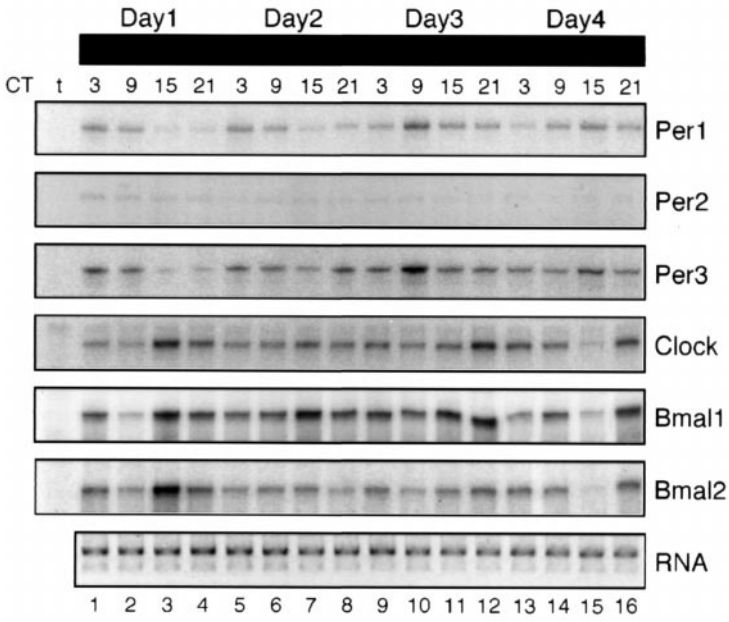

Fig. 4. Duration of circadian oscillation in constant darkness. RNase protection analysis of *Per1*, *Per2*, *Per3*, *Clock*, *Bmal1*, and *Bmal2* gene expression in Z3 cells entrained for 5 days under a 12:12 LD cycle and the placed in constant darkness. RNA was harvested at the indicated CTs for a period of 4 days. The bar above indicates the dark (black) period. tRNA serves as our negative control reaction (t). Relative amounts of total RNA used for each sample are displayed (RNA).

extension of the dark phase (Fig. 5). This inversion caused the LD cycle to shift forward by 12 h. The inversion allowed us to observe that *Per2* is the only central clock component that displays a significant induction in gene activation at the beginning of the first light phase after the cycle inversion (compare lanes 6 and 7). It is possible that *Per2* gene activation initiates entrainment to the new LD cycle and presumably helps the rest of the central clock components to accurately entrain to the new LD cycle. It takes until the second full cycle after the inversion for the rest of the central clock components examined to entrain to the phase set by *Per2* (lanes 11–14). Therefore, *Per2* may play a role in resetting the phase of an established circadian oscillation so that it can entrain to an alternate LD cycle.

Light-Induced Gene Expression During the Subjective Night. If the Z3 cells truly recapitulate the vertebrate clock, photic stimulation during the night phase should result in acute clock gene activation. To determine which central clock components were responsive to photic stimulation during the subjective night, we performed a light pulse time course that spanned ZT16–18 of the night phase. *Per2* displayed robust activation throughout the course of the experiment (Fig. 6), with the 30-min time point being the only exception (lanes 5 and 6). Of interest, the 30-min time point coincides with the normal peak in expression for *Clock*, *Bmal1*, and *Bmal2*. *Per1* only showed an induction at 60 min, whereas *Per3* shows a very modest induction also at the 60-min time point. These results are similar to those obtained in the *Xenopus* retina, in which light exposure was only able to significantly increase *Per2* mRNA levels, and not *Per1*, at all times of the day that were tested (15). Thus, *Per2* is the only *Per* gene that consistently gives a robust gene activation response to photic stimuli throughout the early night phase in the Z3 cells. This data is consistent with the idea that *Per2* may play a role in entraining a circadian oscillation to a new LD cycle. Another

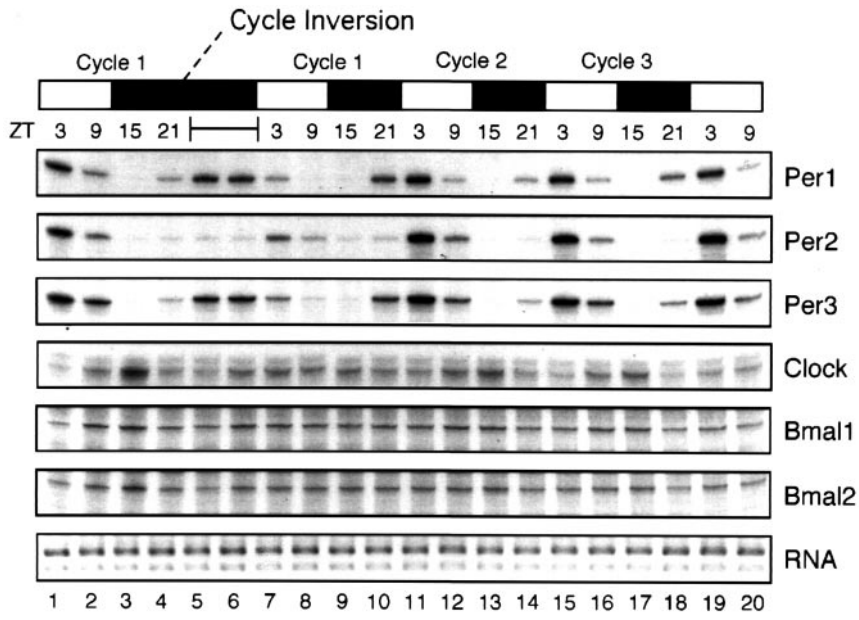

Fig. 5. Entrainment of Z3 cells to an alternate LD cycle. RNase protection analysis of *Per1*, *Per2*, *Per3*, *Clock*, *Bmal1*, and *Bmal2* gene expression in Z3 cells entrained for 5 days under a 12:12 LD cycle. After day 5 of entrainment, the LD cycle was inverted causing a 12-h extension of the dark phase. RNA was harvested at the indicated ZTs. The bar above indicates light (white) and dark (black) periods. tRNA serves as a negative control reaction (t). Relative amounts of total RNA used for each sample are displayed (RNA).

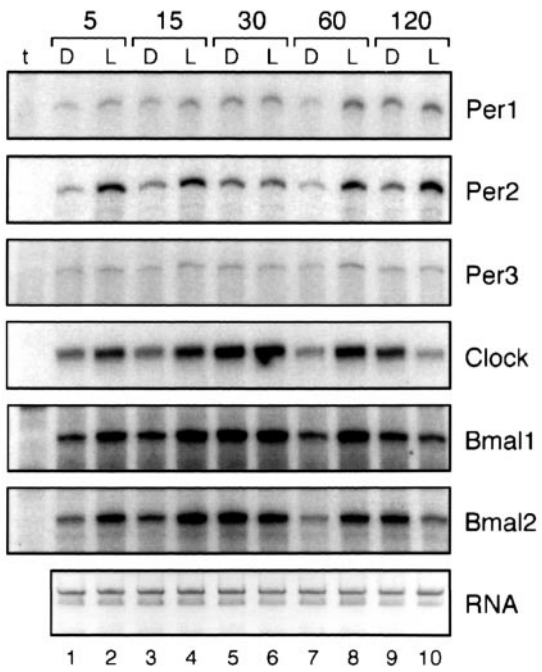

Fig. 6. Light induces gene expression during the subjective night. RNase protection analysis of *Per1*, *Per2*, *Per3*, *Clock*, *Bmal1*, and *Bmal2* gene expression in Z3 cells entrained for 5 days under a 12:12 LD cycle and then given a light pulse. The light pulse began at ZT16 and lasted for 5, 15, 30, 60, and 120 min. RNA was harvested at each time point for light pulsed (L) and nonlight pulsed (D) samples. tRNA serves as a negative control reaction (t). Relative amounts of total RNA used for each sample are displayed (RNA).

interesting observation is that *Per2*, and possibly *Per1* and *Per3*, do not (or are unable to) respond to light stimulation at the same time that the *Clock*, *Bmal1*, and *Bmal2* genes are at their peak expression level (Fig. 6, lanes 5 and 6). This result suggests that there are similar or overlapping components of the transcriptional machinery that are responsible for photic induction of *Per2*, and possibly *Per1* and *Per3*, which are also responsible for the normal oscillation of *Clock* and of the *Bmal* genes.

Clock, *Bmal1*, and *Bmal2* all have very similar responses to the chosen time course. All three genes show induction at the 5-, 15-, and 60-min time points (Fig. 6). They display no light induction during the time of their normal circadian peak in expression, which corresponds to the 30-min time point. They again are light-responsive at 60 min and are suppressed at 120 min. Therefore, *Clock*, *Bmal1*, and *Bmal2* are susceptible to photic induction throughout the early night phase, except when they are already at peak expression levels. The suppression observed at the 120-min time point could be facilitated by the light pulse-dependent production of newly synthesized inhibitory proteins. It is also interesting to note how dynamic the expression profiles of the *Clock* and *Bmal* genes are around their normal time of peak expression (Fig. 6, odd-numbered lanes). The expression patterns do not display a bell-shaped curve, but rather abrupt changes in amplitude and possibly a biphasic peak at the 30- and 120-min time points (Fig. 6, lanes 5 and 9). This observation further demonstrates the complexity of the transcriptional control and regulation necessary to sustain an accurate circadian oscillation.

Discussion

Natural, 24-h LD cycles have been responsible for the evolution of all circadian biological responses (1). Because of millions of years of light-dependent selective pressure, molecular mechanisms have evolved to ensure that also in total darkness, most biological organisms are able to keep an internal clock running to maintain the efficient coordination of physiological processes (9, 10). Here we have described a new cell-based system that will allow more thorough investigation of the light-dependent signaling involved in initiating, entraining, and maintaining robust circadian rhythms and may give insight into how such signaling mechanisms evolved.

The circadian system can be divided into three overlapping components, which are the input pathway, the oscillator, and the output pathway (9, 16, 17). We have demonstrated that Z3 cells are able to recapitulate many functions displayed by input pathways and oscillators. They are able to detect and transfer a photic signal to clock mechanisms contained in each cell. In mammalian systems, the retina is responsible for detecting environmental light and transferring it, as a neuronal signal, via the retino-hypothalamic tract to the mammalian "master" clock located in the suprachiasmatic nucleus within the anterior hypothalamus (2, 18–20). There are many aspects of this complex mammalian photo-signal transduction pathway that could be more easily dissected, both pharmacologically and molecularly, using the Z3 cell line. The Z3 cells also exhibit many features displayed by "master" pacemakers such as the suprachiasmatic nucleus. These characteristics include the precise oscillation of central clock components, oscillation under DD conditions, entrainability to alternate LD cycles, and rapid light-dependent gene activation by a light pulse during the subjective night. It has recently been discovered in a variety of systems, that circadian oscillators exist in peripheral tissues (3–8, 21). Thus, it is a distinct possibility that every cell in a given tissue contains output pathways that regulate the physiology of a specific organ (21). Therefore, the Z3 cells may also become a remarkable tool for studying how clock- and light-regulated mechanisms control output pathways.

An important issue raised by our study is which phototransducing molecules are responsible for circadian light detection and signaling within the zebrafish system. A recent report by Kobayashi *et al.* (22) described the cloning and partial characterization of six cryptochrome (*Cry*) genes. Four of these *Cry* genes show high homology to the mammalian *Cry* genes and were demonstrated to be able to block CLOCK:BMAL-mediated transcription activation (23–25). The remaining two *Cry* genes are much more divergent, and one showed high homology to the *Drosophila* homolog (*dCry*) (26, 27). Unlike mammals and similar to zebrafish (4), *Drosophila* have cell autonomous circadian oscillators that are directly light entrainable (6). In *Drosophila*, it is clear that *dCry* plays a significant role in photoreception and entrainment (26–28). It is thought that *dCry* entrains the circadian clock by stimulating the light-dependent turnover of the TIM (TIMELESS) protein (28, 29). When taken at face value, one might come to the conclusion that a *Cry*-like protein is responsible for light-dependent entrainment of the zebrafish circadian clock. However, until detailed action spectra analyses and functional experiments are done, the possibility still exists that novel opsins, similar to those predicted to be responsible for mammalian photoreception (30–32), are the primary circadian photopigments in zebrafish.

Finally, it is interesting to note that the zebrafish circadian system has overlapping characteristics of both the mammalian and *Drosophila* circadian systems. In *Drosophila*, *Clock* oscillates in a circadian manner, whereas *Cycle* (*Bmal1* homolog) expression is constant (33–35). This is exactly opposite to the mouse mammalian system in which *Bmal* cycles while *Clock* is constant (36, 37). In the zebrafish both *Clock* and *Bmal* oscillate (3, 13). Zebrafish have *Cry* genes similar to both the mammalian and the *Drosophila Cry* genes (22). All three systems have cell autonomous peripheral circadian oscillators (3–8), with only *Drosophila* and zebrafish being directly light-responsive (4, 6, 7). With similarities to both systems, it may not even be too surprising if

the zebrafish circadian system responds to two wavelengths of light: one that corresponds to *Cry* excitation and the other to that of an opsin. Even if this tantalizing scenario does not come to fruition, the Z3 cell-based system will be an invaluable tool for deciphering the molecular mechanisms that regulate light-dependent signaling and circadian rhythms.

We thank Z. Travnickova, D. Whitmore, N. Foulkes, and all members of the Sassone-Corsi laboratory for discussions and help. We also thank K. Barrett and J. Takahashi for providing *Per1* and *Per2* cDNAs, E. Heitz and M. Rastegar for valuable technical assistance, and S. Tan for critical reading of the manuscript. M.P.P. is supported by a postdoctoral fellowship of the Fondation de la Recherche Médicale, A.B.P. by a Rotary predoctoral fellowship, and N.C. by a Canadian Institutes of Health Research postdoctoral fellowship. This work was supported by grants from Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, Centre Hospitalier Universitaire Régional, Fondation de la Recherche Médicale, Université Louis Pasteur, Human Frontier Science Program, and Organon (Akzo/Nobel) and Association pour la Recherche sur le Cancer.

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